Mode of Action of a Herbicide

JOHNSONGRASS AND METHANEARSONIC ACID

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ABSTRACT

Johnsongrass (Sorghum halepense (L.) Pers.) is sensitive to methanearsonate, foliar application resulting in a topkill. Investigation of the pattern of photosynthesis by radioautography revealed an accumulation of malate in methanearsonate-treated leaves. Accumulation of malate was attributed to an inhibition of NADP+-malic enzyme, which was found to be sensitive to sulfhydryl group reagents including arsenosomethane, CH3AsO. Methanearsonate was found to act as an oxidant in the Hill reaction using spinach chloroplasts, the photoproduction being a sulfhydryl group reagent.

These results suggest that methanearsonate inhibits CO2 release from malate in bundle sheath cells, depriving the plant of its source of carbon for sucrose production. The mechanism of inhibition of enzymes sensitive to sulfhydryl group reagents by arsenosomethane is addressed.

Johnsongrass has been described as one of the world's worst weeds (11). It is widespread throughout the United States and is a major pest in the south, infesting cultivated crops, canal banks, and roadsides. Herbicides reported to be effective in control of johnsongrass include the sodium salts of methanearsonic acid (16), mefluidide (20), metribufuron (19), glyphosate (21), dinitroaniline herbicides (18), and dalapon (22). The recent renewal of interest in the metabolism of arsenic (4–6, 10) served to focus our attention on the possible mechanisms of toxicity of methanearsonate in johnsongrass. The sensitivity of johnsongrass to methanearsonate seemed remarkable since the similarly structured bermudagrass was insensitive to the arsenical. Both johnsongrass and bermudagrass are C4 plants wherein CO2 is incorporated into oxalacetate in the mesophyll cells and liberated from malate in bundle sheath cells (8). In the case of johnsongrass (9; see below), malic acid is exported from the mesophyll cells and CO2 is released from malate by an NADP+-malic enzyme in the bundle sheath chloroplasts. Bermudagrass, on the other hand, exports aspartic acid from the mesophyll cell and CO2 is released from malate by a NAD+-malic enzyme in the mitochondria of the bundle sheath cells (3). In this instance, transaminase activities are required and bermudagrass was reported to possess a high level of transaminase activity relative to sugarcane. CO2 released by these decarboxylating systems is subsequently used as a substrate for RuBP carboxylase. The compartmentation of enzymes and the required transport of intermediates which characterizes the C4-dicarboxylic acid pathway is widely varied (8) and we surmised that the difference in sensitivity of johnsongrass and bermudagrass to methanearsonate was related to differences in the mechanisms whereby CO2 is transported to the bundle sheath cells. The toxicity of arsenic-containing compounds is related to the sulfhydryl groups of cells (15, 28) and we noted that malate dehydrogenase of Zea mays was particularly sensitive to O2 (12, 14) and thiols were required for maintenance of activity. It seemed reasonable to presume that methanearsonate exerted its toxic influence on johnsongrass by inhibiting an enzyme with an essential sulfhydryl group that was associated with the metabolism of malate and transport of CO2.

This communication describes results of radiotracer experiments with 14CO2 and enzymic studies of johnsongrass. The results suggest that methanearsonate is reduced to arsenosomethane which inhibits the malic enzyme. Differences in the sensitivities of similar enzymes from johnsongrass and Zea mays to SH reagents are discussed.

MATERIALS AND METHODS

Plant Material. Johnsongrass (Sorghum halepense (L.) Pers.) ecotypes were collected in San Diego, San Joaquin, and Riverside Counties (in California). Plants were maintained in a growth chamber.

Enzyme Preparations. An initial extract of johnsongrass leaves was prepared by homogenizing for 90 s in 4 volumes (w/v) of 50 mM Tricine, 1 mM EDTA, 5 mM MgCl2, pH 8.8. The homogenate was filtered through two layers of cheesecloth, clarified by centrifugation at 3000g for 30 min, and subjected to a buffer exchange operation on Sephadex G-50 equilibrated with the homogenizing buffer.


2 Abbreviations: RuBP, ribulose 1,5-bisphosphate; malate dehydrogenase, l-malate:NAD+ oxidoreductase, EC 1.1.1.37; malic enzyme, l-malate:NADP+ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40; SH reagent, sulfhydryl group reagent; (NBS)0, 5,5-dithiobis(2-nitrobenzoic acid); NBH, 5-thio-2-nitrobenzoic acid; DCIP, 2,6-dichlorophenolindophenol; DTT, thio-1,4-dimercapteto, 2,3-butanediol (reduced DTT).

3 The equilibrium between methylarsonous acid, CH3As(OH)2, and its anhydride form, CH3As=O, appears to favor the anhydride form at neutral pH in aqueous solutions. Various names have been used to describe organic arsenicals such as CH3As=O. The Chemical Abstracts Subject headings lead one to suppose that methylarsonenous acid is its correct name. Methylarsine oxide would appear to be an acceptable name since phenylarsine oxide has been used and named as such in several recent publications and is commercially available as such from Aldrich Chemical Co. We have adopted the usage of Doak and Friedman (7) which names CH3As=O arsenosomethane. While this is convenient for organic arsenicals, extension to the inorganic analog, H—As=O, is difficult.
buffer. The void volume effluent, which was stored in a gas-collecting bottle under an atmosphere of N₂, was used to test the effect of SH reagents on the activity of malate dehydrogenase and malic enzyme.

Ion-exchange chromatography was used to obtain the malic enzyme in a higher state of purity than that afforded by the initial extract. The void volume effluent obtained as described above was concentrated by dialysis against a saturated solution of (NH₄)₂SO₄, salted-out protein being collected by centrifugation and dissolved in 50 mM K-phosphate (pH 7.0 with KOH). Ammonium sulfate was removed by chromatography on Sephadex G-50 equilibrated with 50 mM K-phosphate, the void volume effluent being used to charge a column of DEAE-cellulose. The column was washed with 5 volumes of equilibrating buffer and subjected to gradient elution. The concentration of NaCl in the buffer increased linearly with volume through the column, malic enzyme being eluted with 0.3 M NaCl. The malic enzyme-containing band was stored in a gas-collecting bottle under an atmosphere of N₂. This material was used to study the effect of arsenosomethane on malic enzyme activity.

**Enzyme Assays.** Measurement of the activity of malate dehydrogenase was carried out spectrophotometrically by monitoring the oxidation of NADPH at an analytical wavelength of 340 nm. The reaction was initiated by addition of a suitable aliquot of the enzyme preparation to 1.0 ml 25 mM Tris, 1 mM EDTA, 0.5 mM oxalacetic acid, 0.2 mM NADPH, pH 8.0, in a cuvette with a 1.0-cm light path. The activity of the NADP⁺-malic enzyme was determined by monitoring the reduction of NADP⁺ at an analytical wavelength of 340 nm. The reaction was initiated by addition of a suitable aliquot of the enzyme preparation to 1.0 ml 25 mM Tris, 0.5 mM EDTA, 2.5 mM malate, 0.25 mM NADP⁺, 5 mM MgCl₂, pH 8.0, in a cuvette with a 1.0-cm light path. Absorbance changes were followed with a Beckman DU spectrophotometer fitted with a Gilford photometer unit, multiple sample accessory, and strip chart recorder.

**SH Reagent Inhibition Studies.** SH reagents utilized were N-ethylmaleimide, sodium iodoacetate, 4-chloromercuribenzoic acid, and methanearsonic acid. Ten milligrams of each reagent was dissolved in 2.0 ml of 50 mM Tris, pH 8.0. One-half milliliter of the initial extract was added to each of the SH reagent mixtures and incubated at 0°C for 1 h. A 10-μl aliquot of the initial extract was used to obtain the time course of malate dehydrogenase and malic enzyme-catalyzed reactions as described above. A 50-μl aliquot of the incubation mixtures was used to determine the effect of SH reagents on the time course of the reactions. If SH reagents were without effect on the enzymes, the initial rate obtained with 50-μl aliquots of the incubation mixtures would be identical to that obtained with a 10-μl aliquot of the initial extract because of the dilution factor between the initial extract and the incubation mixture.

**Photo-reduction of Methanearsonate to Arsenosomethane in the Presence of Spinach Chloroplasts.** The time course of photo-reduction of methanearsonate was monitored by the decrease in reactive sulfhydryl groups brought about by condensation of DTTH₂ with the photoproduce, arsenosomethane. Spinach leaves were purchased in a local market and chloroplasts (0.73 mg Chl/ml) were prepared as described by Spencer and Unt (25). Reaction mixtures were prepared in 18-mm diameter test tubes by addition of chloroplasts to 2.0 ml 0.10 M Tris, 3.7 mM DTTH₂, 6.4 mM methanearsonate, pH 7.0. Illumination was provided by a 40-w fluorescent lamp 3.8 cm from the sample. Fifty-microliter aliquots of reaction mixture were withdrawn and added to 5.0 ml 0.050 M K-phosphate, 1.23 mM (NBS)₂, pH 7.0. The A₁₂₅nm was read after 10 min. The molar extinction coefficient for NBSH reported by Riddles et al. (23), 14,150, was used in calculating thiol concentrations. The rate of the photoreduction reaction was studied as a function of methanearsonate concentration. In addition to methanearsonate, the reaction mixture contained 0.07 mM DTTH₂ and spinach chloroplasts equivalent to 0.08 mg of Chl/ml. Thiol groups remaining after 30-min illumination were assayed as described above using a 100-μl aliquot of reaction mixture. Chl was determined by the method of Arnon (1).

**Photo-reduction of 2,6-Dichlorophenol.** Reduction of 65 μM DCIP, 0.10 M Tris, pH 7.0, was monitored at 650 nm. The time course of photoreduction was determined with concentrations of chloroplasts equivalent to 1.1 and 5.5 μg Chl/ml. The reaction mixture was illuminated by a 40-w fluorescent lamp at a distance of 3.8 cm.

**Sensitivity of NADP⁺-Malic Enzyme to Arsenosomethane.** Arsenosomethane was prepared from methylarsine dibromide (Alfa Products, Thiokol/Ventron Division) by alkaline hydrolysis (26). The concentration of an aqueous solution of arsensomethane was determined by reaction with excess DTTH₂ and back titration of remaining DTTH₂ with (NBS)₂. Five-microliter aliquots of arsensomethane were mixed with 100-μl aliquots of enzyme and incubated at 0°C for 30 min. Aliquots of the reaction mixture were withdrawn and assayed as described above. The composition of the buffer solutions was changed as indicated in "results."

**Radioisotope Studies.** Johnsongrass leaves were wet with 1% sodium methanearsonate solution, pH 7.0, and subjected to illumination of 90 ft-c for 24 h. Treated and untreated leaves were then illuminated further in 100 μCi ¹⁴CO₂. After 10 min, the leaves were quickly cut into hot methanol and extracted overnight. Concentrated extracts were analyzed by two-dimensional chromatography on Whatman No. 4 paper. Chromatography in the first dimension was with phenol/water (100:40) and n-butanol/propionic acid/water (142:71:100) was used in the second dimension. Radioautographs were prepared to locate the ³²As-labeled compounds which were measured for radioactivity with a thin-windowed Geiger-Müller counter.

**RESULTS**

**Malic Acid Accumulation in Methanearsonate-Treated Johnsongrass Leaves.** Visible effects of a 24-h pretreatment of johnsongrass leaves with methanearsonate were not apparent, although leaves were markedly desiccated by the end of the second day. The radiograms revealed that sucrose production was greatly diminished and malic acid correspondingly increased in the methanearsonate-treated leaves. They produced malic acid and sucrose in the ratio of 8.1 while in the untreated leaves the ratio was 0.41 (Table I). Methanearsonate treatment inhibited sucrose synthesis 6-fold to 6.6% of the total fixation products, the control leaves incorporated 37% of the ¹⁴CO₂ into sucrose. An experiment with a 60-min ¹⁴CO₂ fixation gave similar results to those obtained with a 10-min ¹⁴CO₂ fixation.

**Sensitivity of Malate Dehydrogenase and NADP⁺-Malic Enzyme to SH Reagents.** Malate dehydrogenase and malic enzyme were present in high concentration in the initial extract. Under the assay conditions used, the activity of the dehydrogenase was approximately 16-fold greater than that of the malic enzyme. Both enzymes exhibited a specific requirement for NADP⁺, the activities with NAD⁺ being nil. Malate dehydrogenase was insensitive to all of the SH reagents tested with the exception of N-ethylmaleimide (Table II). Malic enzyme, on the other hand, was completely inhibited by such reagents. Arsenite was without effect except in combination with 2,3-dimercaptopropanol, where 85% inhibition was observed. Methanearsonate, not known to be a SH reagent, was without effect on either enzyme.

**Sensitivity of NADP⁺-Malic Enzyme to Arsenosomethane and Effect of DTTH₂ on the Enzyme-Inhibitor Complex.** The inhibition at pH 8 was significantly greater than that observed at pH 7, the per cent inhibition of malic enzyme being almost equal although the concentration of arsensomethane was almost 7-fold greater at neutral pH than at the alkaline pH. Addition of DTTH₂...
MECHANISM OF TOPKILL OF JOHNSONGRASS BY METHANEARSONATE

Table I. Incorporation of 14C into Metabolites of Johnsongrass

<table>
<thead>
<tr>
<th>Product</th>
<th>24-h Pretreatment (10-min 14CO2)</th>
<th>30-min Pretreatment (60-min 14CO2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methanearsonate treated</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Methanearsonate treated</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>cpm</td>
<td>cpm</td>
</tr>
<tr>
<td>Malic acid</td>
<td>13,700</td>
<td>4,800</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1,700</td>
<td>11,600</td>
</tr>
<tr>
<td>Alanine</td>
<td>5,300</td>
<td>9,400</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>4,600</td>
<td>4,800</td>
</tr>
<tr>
<td>Malic acid/sucrose</td>
<td>8.1</td>
<td>0.41</td>
</tr>
</tbody>
</table>

* Leaves were illuminated after wetting with methanearsonate.

Table II. Effect of Sulfhydryl Group Reagents on the Reactivity of Malate Dehydrogenase and Malic Enzyme of Johnsongrass

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Malate dehydrogenase</th>
<th>Malic enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>0.020 M Iodoacetate</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.022 M Methanearsonate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.032 M N-Ethylmaleimide</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>0.011 M 4-Chloromercurobenzoate</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.0005 m 2,3-Dimercapto-1-propanol (BAL)</td>
<td>0</td>
<td>10% activation</td>
</tr>
<tr>
<td>0.005 M Arsenite</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.005 M Arsenite-BAL (1:1)</td>
<td>0</td>
<td>85</td>
</tr>
</tbody>
</table>

Table III. Inhibition of Johnsongrass Malic Enzyme by Arsenosomethane, CH₃AsO

Response of the enzyme-inhibitor complex to DTTH₂ and Mercaptoethanol.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Inhibition by CH₃AsO</th>
<th>Reactivation by DTTH₂</th>
<th>Mercaptoethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>0.025 M Tris, pH 8</td>
<td>80</td>
<td>80*</td>
<td></td>
</tr>
<tr>
<td>0.0001 m CH₃AsO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 M Pi, pH 7</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001 m CH₃AsO</td>
<td>93</td>
<td>90*</td>
<td>0</td>
</tr>
<tr>
<td>0.025 m Tris, pH 8</td>
<td>100*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* DTTH₂ added to a final concentration of 0.7 mM.

** DTTH₂ added to a final concentration of 4 mM. A heavy precipitate, formed by formation of the binary addition product of DTTH₂ and CH₃AsO, was removed by centrifugation.

directly to the inhibited mixture in the presence of substrates resulted in an immediate recovery of 80% of the activity of the uninhibited control. Increasing the concentration of inhibitor to 1 mM resulted in a stronger inhibition of the activity of the malic enzyme, an inhibition which could be substantially relieved by addition of excess DTTH₂. The stabilizing effect of mercaptoethanol on the enzyme inhibitor complex was demonstrated by the complete inhibition of malic enzyme observed when this monothiol was added to the mixture of enzyme and inhibitor. A prior addition of mercaptoethanol blocked completely the ability to regenerate active enzyme with DTTH₂ (Table III).

Photochemical Formation of a SH Reagent from Methanearsonic Acid in the Presence of Spinach Chloroplasts. The conversion of methanearsonate to a SH reagent was carried out in the presence of excess DTTH₂. Formation of a SH reagent would result in a decrease in the number of sulfhydryl groups available for reaction with (NBS)₂. Progress curves for light-dependent loss of DTTH₂ are illustrated in Figure 1 for chloroplast suspensions equivalent to 0.021 and 0.146 mg Chl/ml. The rate of consumption of DTTH₂ with the lower concentration of Chl was nearly linear over a time course of 110 min and equal to 6.1 μmol DTTH₂/mg Chl/h, while that obtained at a concentration of Chl 7-fold higher was decreasing continuously with time. The initial rate of consumption of DTTH₂ was 7.1 μmol/mg Chl/h. Measurement of the loss of DTTH₂ during a 30-min incubation showed that the rate of the reaction was directly proportional to Chl concentration at values less than 0.04 mg/ml and decreased continuously as the concentration rose above 0.04 mg/ml (results not presented).

The rate of photoreduction of methanearsonate was also found to depend on the concentration of methanearsonate. An approximately linear dependence of the rate on methanearsonate was observed, although the curve did not possess a zero intercept on the y axis (Fig. 2). Graphic analysis of the rate data according to the Lineweaver-Burk reciprocal modification of the Michaelis-Menten equation gave a satisfactory fit of the data to a straight line (reciprocal plot not illustrated). A least squares fit of the data to a straight line yielded a value of Kₘ = 6.3 mM (correlation coefficient = 0.976).

Photoreduction of DCIP was measured using chloroplast concentrations of 1.1 and 5.5 μg Chl/ml. The observed rates were 139 and 163 μmol/mg Chl/h, respectively.
**DISCUSSION**

Radiolabeling experiments point directly at the malic enzyme as the site of methanearsonate-mediated toxicity in johnsongrass. Accumulation of malate, relative to sucrose, was increased 20-fold over that of the control experiment. Synthesis of sucrose requires CO₂ provided by the action of the malic enzyme on malate in the bundle sheath chloroplasts. Inhibition of the malic enzyme, suggested by the accumulation of malate, is also consistent with the observed sensitivity of the malic enzyme to SH reagents in general and arsenosomethane in particular. The accumulation of malate was puzzling in view of the results of earlier studies of the SH reagent sensitivity of malate dehydrogenase and malic enzyme of *Zea mays* (14). Malate dehydrogenase was found to be sensitive to SH reagents while the malic enzyme was not, leading to the expectation that oxalacetate would accumulate. The finding that the SH reagent sensitivity of the last two enzymes of the CO₂ transport system of johnsongrass was the reverse of that observed in *Zea mays* conserved the hypothesis of Voegelin et al. (28) that arsenicals react with the sulphydryl groups of cells as well as accounting for our results.

The sensitivity of malate dehydrogenase from *Zea mays* to SH reagents may be compared with that of the malic enzyme from johnsongrass. In the case of malate dehydrogenase, SH reagent sensitivity resides in a vicinal dithiol group. The enzyme is active in light and inactive in darkness, conversion between the active and inactive forms being controlled by a thioredoxin, which reversibly reduces a cysteine residue to the vicinal dithiol group (2). Thioredoxin is linked to the photosynthetic electron transport chain by NADPH-thioredoxin reductase, a pyridine nucleotide-disulfide oxidoreductase which utilizes a FAD moiety in transferring an electron pair from NADPH to a disulfide bridge (30). Malate dehydrogenase is particularly sensitive to O₂ and addition of DTTH₂ to inactive enzyme restores full activity. The sensitivity of malate dehydrogenase to SH reagents, then, is due to reaction with one or both of the thiol moieties formed by reduction of the cysteine residue. The malic enzyme of johnsongrass, on the other hand, was not particularly sensitive to O₂ and treatment with DTTH₂ did not result in an increase in activity. Furthermore, inhibition by arsenosomethane was readily reversed by low concentrations of DTTH₂. These observations suggest, as a basis for further experimentation, that SH reagent sensitivity of the johnsongrass malic enzyme is due to the presence of a single sulphydryl group. The malic enzyme from *Zea mays* was not reported to be sensitive to SH reagents and it is reasonable to expect the catalytic mechanisms to be conserved. The sensitivity of johnsongrass malic enzyme to arsenosomethane, then, could be due to a cysteiny1 residue close to the active site, derivatization of the SH group preventing approach of the substrate to the active site. The SH reagent sensitivity of malate dehydrogenase from *Zea mays* seems to be a consequence of the mechanism for regulating its activity by light whereas the sensitivity of the johnsongrass malic enzyme to SH reagents appears to be only coincidental.

The accumulation of malate in methanearsonate-treated leaves of johnsongrass suggests that the efficacy of arsenosomethane *in vivo* may exceed that observed *in vitro* with malic enzyme. A 20-fold increase in the accumulation of malate with respect to sucrose would appear to require a concentration of arsenosomethane greater than 0.1 mM, which afforded only 80% inhibition of malic enzyme at pH 8.0. Such an accumulation of inhibitor in the leaf would be remarkable in view of the small amount of methanearsonate applied to the leaf. The following considerations suggest, however, that arsenosomethane may be more effective *in vivo* than suspected from the malic enzyme experiments conducted *in vitro*.

(a) Upon diffusion of methanearsonate into the vascular system, it will remain in close contact with bundle sheath cells. Johnsongrass possesses Kranz anatomy (17) and the uptake of methanearsonate by the bundle sheath cells and photoreduction by the chloroplasts of these cells would lead to the formation of inhibitor at the very site of inhibition. (b) Arsenosomethane may react more strongly with malic enzyme *in vivo* than *in vitro* due to its high degree of solubility in nonaqueous solvents. Malic enzyme may be closely associated with membranes in the chloroplast and the reaction of arsenosomethane with sulphydryl groups would be favored by nonpolar environments. In this regard, we note that the sensitivity of the ATPase of intact mitochondria to arsenosobenzene (13, 24, 27) and arsenosomethane (F. C. Knowles and A. F. Knowles, unpublished results) appears to be due to a single sulphydryl group on the F₆ subunit. A stimulation of the membrane-bound ATPase was not obtained with SH reagent such as pCMB and (NBS), which require a polar environment for reaction. (c) Other factors may exist in the chloroplast which enhance inhibition by arsenosomethane. The formation of an association complex between an enzymic sulphydryl group and arsenosomethane leaves arsenic with half of its capacity for sulfur satisfied. Condensation of a monothiol with the enzyme-inhibitor complex has the effect of increasing the association constant of the inhibitor for the enzyme. Such enhancement of inhibition is well documented with organic arsenicals (29). Treatment of intact mitochondria with arsenosobenzene stimulates the ATPase, an effect which can be reversed by DTTH₂ (13, 24). Addition of mercaptothanol before addition of DTTH₂ blocks restoration of coupling. A mechanism to account for potentiation of inhibition is illustrated in Figure 3. (d) The role played by the malic enzyme in johnsongrass is exceptionally important since assimilation of carbon is impossible without release of CO₂ from malic acid. The consequences of inhibition of this enzyme, then, are perhaps more far reaching than would otherwise be the case. There is no possibility for a metabolic adaptation to a lack of substrate. Thus, we may conclude from the foregoing discussion that the accumulation of malate in methanearsonate-treated leaves of johnsongrass is consistent with inhibition of malic enzyme by an inhibitor formed photochemically from methanearsonate.

The results obtained with spinach chloroplasts demonstrate clearly that a sulphydryl group reagent may be formed from methanearsenic acid. DTTH₂ persisted in solutions of methanearsonate and chloroplasts in darkness and only disappeared when the solution was illuminated. The reactions proposed to account for disappearance of DTTH₂ are as follows

\[
\begin{align*}
\text{CH}_3\text{AsO}_3^{2-} + 2\text{H}^+ + 2e^- & = \text{CH}_3\text{AsO} + 20\text{H}^-
\end{align*}
\]

(a)

\[
\begin{align*}
\text{CH}_3\text{AsO} + \text{DTTH}_2 & = \text{CH}_3\text{As} + \text{H}_2\text{O}
\end{align*}
\]

(b)

\[
\begin{align*}
\text{SCNCH}_2\text{OH}
\end{align*}
\]

(c)

The reactivity of arsenic (+5), represented by CH₃AsO and HAsO₂,
as opposed to arsenic (+3), represented by methanearsonic acid and arsenite, is well established. The formation of a stable bond between arsenic and organic sulfhydryl groups requires arsenic in the formal oxidation state of +1 and the light-dependent loss of DTTH2 implies that methanarsonate was reduced to arsenosomethane. The reaction written in Equation 2, then, is well established and the fact of its existence relies entirely on the existence of the half-reaction written in Equation 1.

Equation 1 is a half-reaction for the reduction of methanarsonate by two electrons, the reducing equivalents derived from photochemical cleavage of water being intercepted from the electron transport chain. It is not unreasonable to presume that methanarsonate is a competitive substrate for NADPH-thioredoxin reductase. An analogy to this type of redox reaction was provided by (a) the recent finding that DTTH2 will reduce arsenite to arsonous acid and (b) the inference that arsenite will accept electrons from the incipient dithiol grouping of diaphorase in the presence of NADH (15). The ability of a dithiol of low potential to reduce As (+3) to As (+1), then, seems well established mechanistically and such reductants are present in illuminated chloroplasts. The efficacy of methanarsonate as an oxidant in the Hill reaction was approximately one-tenth that of DCIP, indicating that the reduction to arsenosomethane was a facile reaction. The Hill reaction, then, can account for the formation of an enzyme-inhibitory form of arsenic. We may infer that such reactions have occurred in johnsongrass but the evidence is indirect, being that an enzyme with a sulfhydryl group essential for activity became inhibited in the presence of arsenicals.

The results obtained above suggest a mode of action of methanarsonate in johnsongrass. The johnsongrass malic enzyme, which catalyzes the release of CO2 from malic acid in bundle sheath chloroplasts, is a SH reagent-sensitive enzyme which is inhibited by a SH reagent, arsenosomethane, formed by an in vivo Hill reaction. Johnsongrass possesses an elaboration of the photosynthetic cycle whereby CO2 is incorporated into oxalacetate in mesophyll cells and transported to bundle sheath cells as malate. Assimilation of CO2 for photosynthesis of carbon compounds by johnsongrass depends on a sequence of enzyme-catalyzed reactions, a process which is subject to both regulation and inhibition. The site of inhibition in johnsongrass is particularly critical for the plant insofar as the consequence of inhibition deprives the plant of its carbon source.

In concluding, we may address the question of the effect of methanarsonate on plants other than johnsongrass where similar mechanisms for formation of arsenosomethane exist without the coincidental presence of a key metabolic reaction subject to inhibition. The reactivity of arsenosomethane toward any enzymic sulfhydryl group would be similar to that described above for the malic enzyme. The degree of inhibition would decrease as the path between the site of formation of the inhibitor and the site at which the inhibitor acts increases. Thus, we may expect the degree of inhibition to decrease and the consequences to be less dramatic than obtained with johnsongrass. Light-mediated activation of enzymes requires thioredoxin reductases, thioredoxins, and target enzymes (2). These moieties all contain vicinal dithiol groupings which can condense with arsenosomethane to form arsenic-bridged disulfide bonds of high stability. These light activation systems, furthermore, are localized in chloroplasts and are physically close to the site of formation of arsenosomethane. We can expect, then, that the ability of a plant to respond to a change in illumination by sulfhydryl-mediated modification of enzyme structure would be impaired. The sequence of reactions involving light activation and the possible interactions of these intermediates with arsenosomethane are summarized in Figure 4.
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